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Analysis of differential immune responses induced by innate and adaptive immunity following transplantation

HONGZHEN HE,* JAMES R. STONE† & DAVID L. PERKINS* **Laboratory of Molecular Immunology, Department of Medicine and †Department of Pathology, Brigham & Women's Hospital, Harvard Medical School, Boston, AM, USA*

SUMMARY

The roles of innate and adaptive immunity in allograft rejection remain incompletely understood. Previous studies analysing lymphocyte deficient or syngeneic graft recipients have identified subsets of inflammatory chemokines and cytokines induced by antigen independent mechanisms. In the current study, we analysed a panel of 60 inflammatory parameters including serum cytokines, intragraft chemokines and cytokines, receptors, and cellular markers. Our results confirmed the up-regulation of a subset of markers by innate mechanisms and also identified a subset of parameters up-regulated only in the context of an adaptive response. Thus, we successfully differentiated markers of the innate and adaptive phases of rejection. Current paradigms emphasize that innate signals can promote a subsequent adaptive response. Interestingly, in our studies, expression of the markers induced by innate mechanisms was markedly amplified in the allogeneic, but not syngeneic or lymphocyte deficient, recipients. These results suggest that inflammatory mediators can have functional overlap between the innate and adaptive responses, and that the adaptive component of the rejection process amplifies the innate response by positive feedback regulation.

INTRODUCTION

The immune response is composed of innate and adaptive components. The innate response is phylogenetically primitive and occurs earlier during a response to infection or injury, whereas the adaptive response evolved more recently within the vertebrates and occurs later during an immune response.[1] The innate response is antigen independent and uses pattern recognition receptors to respond to conserved molecular motifs.[2,3] The specificity of pattern recognition receptors, which comprise a small number of invariant receptors that are encoded in the germline, has been selected during evolution to recognize molecules produced by infectious agents including bacteria, viruses, and fungi or induced by stress.

The adaptive response is antigen specific and uses the T- and B-cell receptors (TCR and BCR, respectively) to recognize antigen. The TCR and BCR are somatically rearranged during development by random processes to generate large numbers of unique receptors that are capable of recognizing a diverse array of antigens.[4] This process is mediated by the recombinase activating genes (RAG).[5] In current paradigms of immunity, an important function of innate immunity is to generate a rapid first line defence and promote the activation of the adaptive response. In this model, triggering of pattern recognition receptors creates a proinflammatory milieu that activates T- and B-cell responses of adaptive immunity.[6,7] In a well-studied model of sepsis, lipopolysaccharide, a component of gram negative bacterial cell walls, triggers the Toll-like receptor-4 (TLR4) pattern recognition receptor to produce proinflammatory conditions that promote the activation of an adaptive immune response.[8] In our study, we used three experimental groups including RAG-deficient (alymphoid) and syngeneic recipients that lacked adaptive immunity, and allogeneic recipients that generated a wildtype alloimmune response.

In transplantation, the role of innate immunity remains poorly understood. In experimental models of transplantation, there is no evidence for pathogenic infection suggesting that if innate immunity is activated it is triggered by non-infectious stimuli. Consistent with this observation, recent studies have identified non-infectious stimuli of innate signals including stress proteins, such as heat shock protein 60, and fibronectin that are capable of signalling via Toll pathways.[9–11] In addition, other receptors not in the TLR family likely trigger innate responses.[12] Following transplantation, antigen independent stimuli including ischaemia, reperfusion, surgical injury, systemic

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Correspondence: Dr D. L. Perkins, Brigham & Women's Hospital, PBB-170, 75 Francis St, Boston, MA 02115, USA. E-mail: dperkins@rics.bwh.harvard.edu

stress, and brain death may contribute to the initiation of an inflammatory response.[13,14]

In a previous study, our laboratory reported the analysis of a subset of inflammatory mediators induced following transplantation by innate responses using antigen independent mechanisms;[15] in the current study we identified an additional subset of chemokines, cytokines and other inflammatory markers induced during rejection only in the context of an adaptive immune response. Previous reports have shown that innate responses up-regulate cytokines, for example interleukin (IL)-12, and costimulatory molecules, for example B7-2, that promote the activation of subsequent adaptive immune responses. We postulated that reciprocal regulation of innate responses by adaptive immunity are also important in the development of an *in vivo* alloimmune response. To detect evidence of adaptive regulation of the response initiated by innate immunity, our experimental design involved the analysis of a panel of 60 inflammatory parameters, including the subsets up-regulated during either the innate or adaptive components of the rejection response. Based on our analysis of this large panel of inflammatory markers, our results indicate a robust amplification by the adaptive response of the expression of multiple parameters that were initially induced by antigen independent mechanisms. Thus, our results support a model in which inflammatory mediators are induced by innate mechanisms immediately following transplantation, and that the subsequent adaptive response amplifies the original innate response and also produces additional inflammatory mediators.

MATERIALS AND METHODS

Vascularized heterotopic cardiac transplantation

Murine hearts were transplanted as previously described.[16] Briefly, hearts were harvested from freshly sacrificed donors and immediately transplanted into 8–12-week-old-recipients which were anaesthetized via intraperitoneal (i.p.) injection with 60 mg/kg of pentobarbital sodium. The donor aorta was attached to the recipient abdominal aorta by end to side anastomosis, and the donor pulmonary artery was attached to the recipient vena cava by end to side anastomosis. All surgical procedures were completed in less than 60 min from the time that the donor heart was harvested. Donor hearts that did not beat immediately after reperfusion or stopped within two days after transplantation were excluded (>95% of all grafts functioned at 2 days after transplantation). Donor grafts were harvested at 24 hr after transplantation and divided into equal sections for preparation of RNA and tissue sections for histology.

Mice

Eight- to 12-week-old-male mice including BALB/cByJ (BALB/c) (H-2^d), C57BL/6J (B6) (H-2^b), C57BL/6J-Rag-1^{tm1Mom} (B6-Rag KO) (H-2^b) (JAX, Bar Harbor, ME), and BALB/c-AnNTac-Rag2^{tm1}N12 (BALB/c-Rag KO) (H-2^d) (Taconic, Germantown, NY) were used as donor and recipients in the transplant experiments. Mice are maintained in vented racks with constant temperature and humidity in our animal facility under virus antibody free conditions.

Enzyme-linked immunosorbent assay (ELISA)

Cytokines tumour necrosis factor-α (TNF-α), IL-6, interferon (IFN)-γ and IL-1β, were evaluated using Quantikine M immunoassay (R & D Systems Inc., Minneapolis MN) as per the manufacturer's directions. Briefly, the specific antibody for each cytokine was precoated in microtitre wells. Serum was analysed from four separate graft recipients analysed in triplicate. One hundred μl of the serum sample or cytokine standard was incubated overnight at 4°. Based on our previous studies to establish reproducibility, the samples for IL-1β and IFN-γ were harvested for evaluation in the IL-6 and TNF-α tests, respectively. An enzyme-linked polyconal antibody specific for the particular cytokine was added to the wells and incubated for 2 hr. After washing, the substrate tetramethylbenzidine plus hydrogen peroxide was added and incubated 30 min at room temperature. After adding a stop solution of dilute hydrochloric acid, the optical density was measured with a Emax microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm wavelength, along with a correction reading at 540 nm wavelength for optical interference by the microtitre plate. Triplicate readings of the serum samples and duplicate readings of each standard were averaged after subtracting the background standard optical density. Final sample calculations were based on a regression analysis of the log of the final optical density vs. the log of standard dilutions and reported as pg ml. The sensitivity of detection is 3·0, 3·1, 2·0, and 5·1 pg/ml for IL-1β, IL-6, TNF-α, and IFN-γ, respectively; all cytokines were below the level of detection in untransplanted normal control mice.

Ribonuclease protection assay (RPA)

Chemokine, chemokine receptor and CD marker expression was analysed by RPA. Briefly, total RNA was isolated from hearts using RNAzol and analysed using the RiboQuant Multi-Probe RNAse Protection Assay (RPA) System (Pharmingen, San Diego, CA). Fifteen μg of RNA was used as per hybridization and RNase reaction with the templates mCK-5 (macrophage inhibition factor (MIF), regulated on activation, normal, T-cell expressed, and secreted (RANTES), eotaxin, macrophage inflammatory protein (MIP)-1β, MIP-1α, MIP-2, interferon inducible protein-10 (IP-10), and monocyte chemoattractant protein-1 (MCP-1)), mCR-5 (CCR1, 1β, 4, 5, and 2), a custom template (CXCR2, 3, 4, 5 and CCR6, 8α, and 8β) and mCD-1 (TCRoad, TCRα, CD3ϵ, CD4, CD8α, CD8β, CD19, F4/80, CD45; Pharmingen, San Diego, CA). The IP-10 template detects the C57BL/6 allele.[17] The protocol was modified by labelling probes with ^{35}S. After hybridization with the ^{35}S-labelled probes, the samples were treated with RNase and purified according to the manufacturer's protocol. The protected probes were electrophoresed on a denaturing 5% polyacrylamide gel. The gels were exposed in a Molecular Dynamics phosphorimager. The identity of each protected fragment was established by analysing its migration distance against a standard curve of the migration distance versus the log nucleotide length for each undigested probe. Samples were normalized to the housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH). Protected bands were quantitated by densitometry analysis using ImageQuant software (Molecular Dynamics).

Histology

Recipient native hearts and donor transplanted hearts were harvested 1 day after transplantation and fixed in 10% neutral buffered formalin. After dehydration and paraffin embedding, 5–6 μm thick sections were routinely stained with haematoxylin and eosin. Multiple sections were examined for each heart and the extent of rejection (grade 0–4) and ischemia (grade 0–4) were quantified using a modified International Society of Heart and Lung Transplantation grading scale.[18] Any graft hearts with ischemia score >1 were excluded from analysis.

Table 1. Experimental groups

Group	Donor	H-2	Recipient	H-2	MST (days)
Syngeneic	B6	b	B6	b	>100
Alymphoid	BALB/c-RAG	d	B6-RAG	b	>100
Allogeneic	BALB/c	d	B6	b	7·8

The 'syngeneic' group has B6 donors and recipients. The 'alymphoid' group has RAG deficient BALB/c-RAG donor and B6–RAG recipient strains with a complete H-2 class I and II mismatch. The 'allogeneic' group has wildtype BALB/c donor and C57BL/6 recipient strains that have a complete H-2 Class I and II mismatch. MST = mean survival time. $n = 6$ per group. Graft survival data were calculated as mean ± SD. $^*P < 0{\cdot}05$.

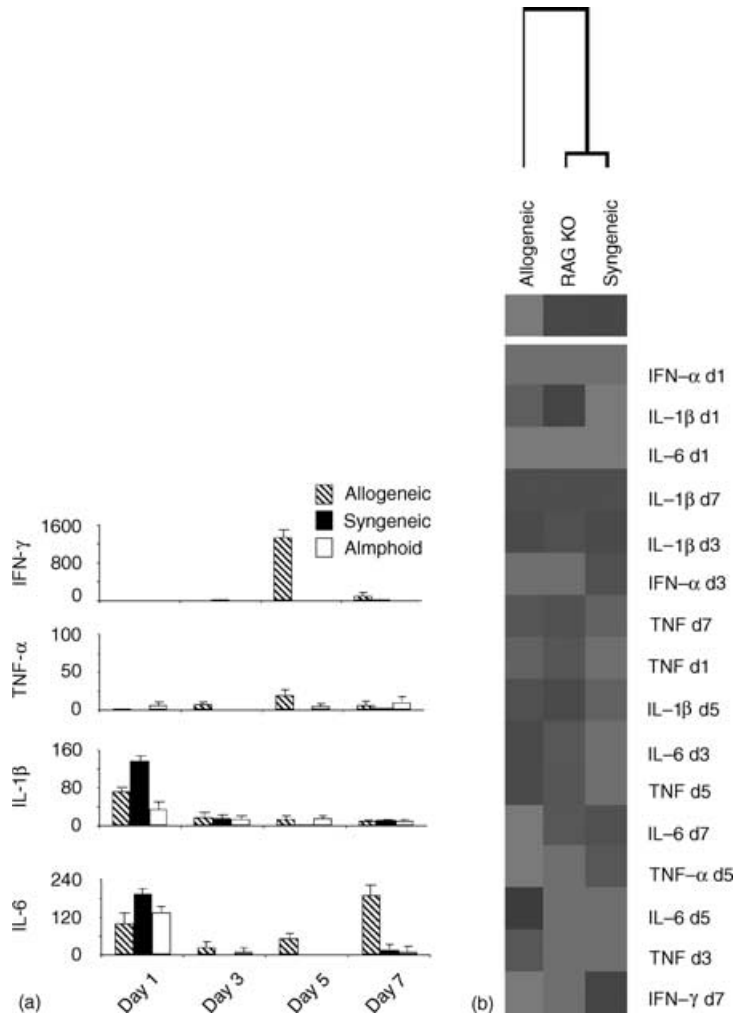

Figure 1. Serum cytokines. (a) The levels of TNF-α, IL-1β, IL-6, and IFN-γ (pg/ml) were determined by ELISA on days 1, 3, 5, and 7 following transplantation in the allogeneic (hatched), syngeneic (filled), and alymphoid (open) groups. (b) Dendrogram of kinetic analysis of cytokine profile in transplant recipients. The level of serum cytokine expression was determined at days 1, 3, 5 and 7 following transplantation by ELISA and analysed by Cluster and Tree software in allogeneic, alymphoid, and syngeneic graft recipients. Control serums are from untransplanted B6 mice. Using greyscale representation, the lighter the colour the higher the expression. The degree of dissimilarity is proportional to the total length of the vertical axis between groups.

Statistics

Cluster analysis was performed using Cluster and TreeView[19] (courtesy of M Eisen, Lawrence Livermore Radiation Laboratory, Berkeley, CA). Cytokine values were analysed by Cluster using the hierarchical clustering algorithm with complete linkage clustering. Briefly, dissimilarity is determined by calculation of the Pearson correlation coefficient between each series of values from each experimental group.[19] After processing, the dendrogram was visualized by TreeView. Serum cytokines were calculated as the mean plus standard deviation of quadruplicate values. Differential expression of mRNA determined by RPA 24 hr following transplantation in the native and graft hearts of the syngeneic, alymphoid, and allogeneic recipients by two factor analysis of variance (ANOVA). Statistical significance of variances was calculated for $P < 0{\cdot}05$ by F-test. Differential expression of mRNA determined by RPA on days 1, 3, 5, and 7 in syngeneic, alymphoid, and allogeneic samples and untransplanted control heart RNA was analysed by two factor analysis of variance (ANOVA). Statistical significance of variances was calculated for $P < 0{\cdot}05$ by F-test.

RESULTS

To investigate the role of the innate and adaptive immune responses following transplantation, we performed a kinetic analysis of the rejection responses in three experimental groups including BALB/c-RAG KO → B6-RAG KO (alymphoid), BALB/c → B6 (allogeneic), and B6 → B6 (syngeneic) (Table 1). In the alymphoid group, both the donor and recipient strains lack functional T and B cells eliminating any antigen-specific adaptive immune response; however, all innate immune components, including a potential natural killer cell response are intact. In the syngeneic group, both natural killer cell responses and adaptive immunity should not be stimulated due to genetic identity, whereas in the allogeneic group both innate and adaptive responses are operative.

We first analysed the levels of the acute phase cytokines TNF-α, IL-1β, IL-6 plus IFN-γ (Fig. 1a). All three experimental groups had increased serum levels of IL-1β and IL-6 on day 1 following transplantation indicating that these cytokines did not require an adaptive immune response to be up-regulated. The

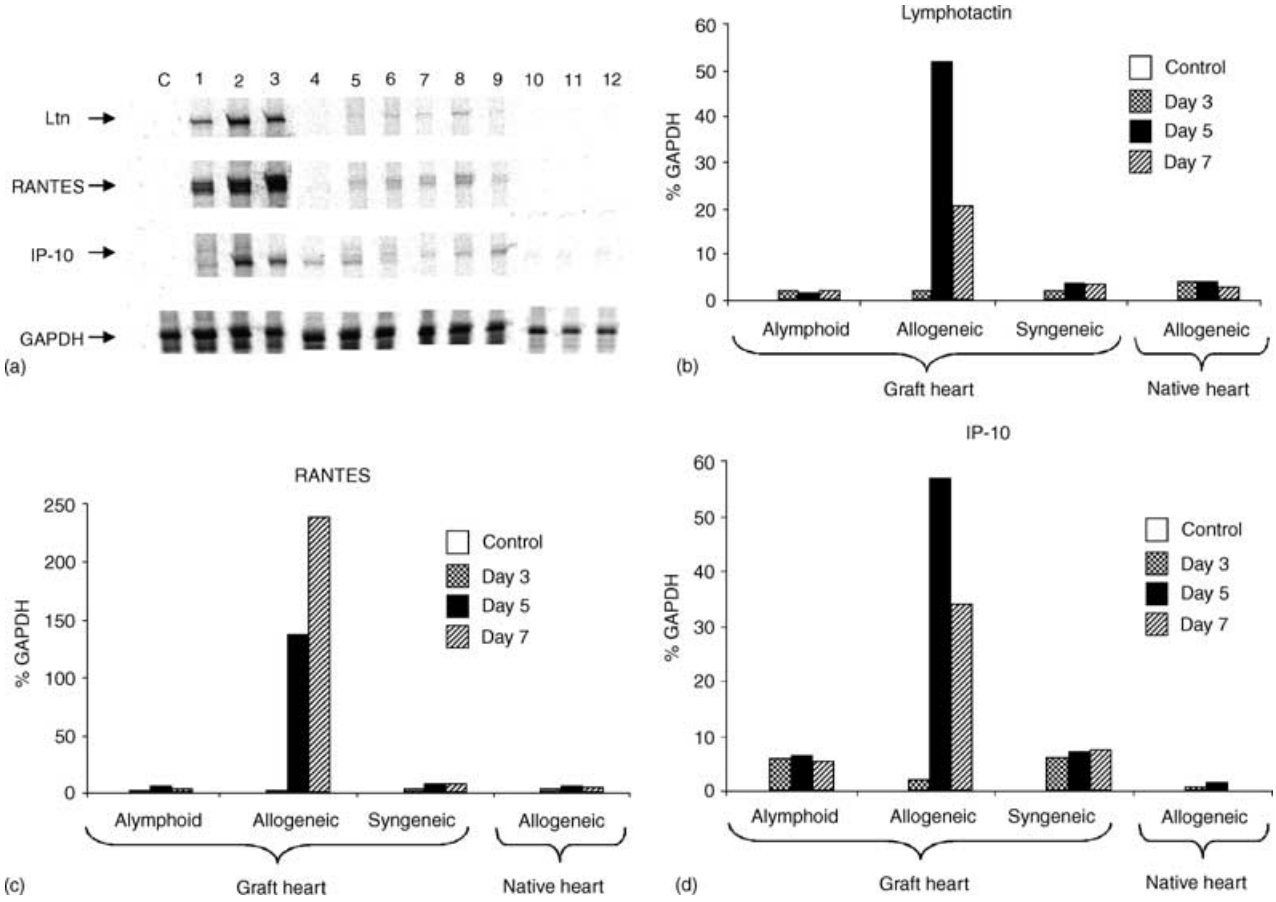

Figure 2. Late expression of chemokines. (a) Total RNA was isolated from hearts using RNAzol. RPA analysis was performed using the RiboQuant Multi-Probe RNAse Protection Assay (RPA) System (Pharmingen, San Diego, CA). RNA was hybridized with ^{35}S-labelled template including RANTES, Ltn and IP-10. Untransplanted control heart (lane C), allogeneic graft hearts d 3,5,7 (lanes 1–3), alymphoid graft hearts d 3,5,7 (lanes 4–7), syngeneic graft hearts d 3,5,7 (lanes 8–10), and allogeneic native hearts d 3,5,7 (lanes 11–12). Densitometry analysis was performed using ImageQuant software (Molecular Dynamics), and expression in control (open), day 3 (checks), day 5 (filled) and day 7 (crosshatch) of (b) Ltn, (c) RANTES, and (d) IP-10, were normalized to the housekeeping gene GAPDH. The data shown are representative of through independent experiments.

levels of both IL-1β and IL-6 decreased on days 3 and 5. However, on day 7 IL-6 increased markedly in the allogeneic, but not the alymphoid or syngeneic groups, suggesting that a second phase of IL-6 production was induced during the rejection process. In addition, IFN-γ was up-regulated only in the allogeneic group on day 5 following transplant. TNF-α was expressed at low levels at all time points analysed; however, this does not exclude the possibility it is increased at time points not analysed. Using hierarchical cluster analysis, our data of serum cytokine levels differentiated the allogeneic group that rapidly rejects grafts from the alymphoid and syngeneic groups (Fig. 1b). Specifically, IFN-γ and the late phase of IL-6 are induced only in the presence of an adaptive response.

We identified a subset of chemokines including lymphotoxin (Ltn), RANTES, and IP-10 that were up-regulated predominantly in the allogeneic group, but not the alymphoid or syngeneic groups (Fig. 2a). By day 5, Ltn gene expression exceeded 50% of GAPDH on day 5 (Fig. 2b). Similarly, RANTES gene expression increased to extremely high levels reaching 240% of GAPDH on day 7 (Fig. 2c). In the alymphoid and syngeneic groups, however, there were no detectable increases in the expression of Ltn or RANTES. We did detect low levels of IP-10 on day 3 in all three groups; however, IP-10 only significantly increased at days 5 and 7 in the allogeneic group (Fig. 2d). In the native heart samples, we did not detect increases in Ltn, RANTES, or IP-10. These results show that large increases in the level of expression of Ltn and RANTES occur only in the presence of an adaptive immune response.

We also analysed the level of expression of the chemokines MIP-1α, MIP-1β, MIP-2, and MCP-1, which are readily detected in the alymphoid group at day 3 following transplantation (Fig. 3a). As expected, all four of these chemokines were up-regulated in all three experimental groups; however, the pattern and magnitude of expression was different in the allogeneic group (Fig. 3b–e). This is likely to be a result of positive feedback by the adaptive immune response in the allogeneic group. On day 3 following transplantation, the magnitude of expression of most of these chemokines was comparable in all through groups; however, by days 5 and 7 after transplantation the levels of all four chemokines were markedly increased in the allogeneic group as compared to both the alymphoid and syngeneic groups. Only low levels of these chemokines were detected in the native heart samples. These results indicate that an adaptive immune response is not necessary for the induction of MIP-1α, MIP-1β, MIP-2, and MCP-1, and in fact, at early time point after transplantation, the level of expression is not affected by the presence of an adaptive response. The up-regulation of these chemokines at later time points, however, occurs only in the context of an adaptive immune response.

Additional chemokines showed only minor changes in the level of expression. For example, MIF, which is constitutively expressed in untransplanted fresh heart tissue at approximately 20–25% of GAPDH, showed only minor changes in expression (Fig. 3f). Also, eotaxin, which is not constitutively expressed in heart tissue, was up-regulated to low levels not exceeding 5% of GAPDH (Fig. 3g). In summary, our results demonstrate differential recruitment of inflammatory cells and regulation of individual chemokines and receptors by the innate and adaptive components of the immune response after transplantation.

We analysed graft histology in order to assess cellular infiltration and other markers of rejection, including myocyte necrosis. (Fig. 4). At day 3, graft hearts from all three experimental groups showed grade 0 rejection with mild focal mononuclear cell infiltration, but without myocyte necrosis (Fig. 4d–f). Untransplanted control hearts showed no evidence of infiltration or rejection (Fig. 4a). As expected, the allogeneic grafts, which had a mean survival time of 7·8 days, showed increasing degrees of infiltration with grade 4 rejection including myocyte necrosis evident by day 7 (Fig. 4g). At this time the alymphoid and syngeneic grafts had no evidence of rejection without myocyte necrosis (Fig. 4h, i). The native hearts of the allogeneic group (Fig. 4b, c) and alymphoid and syngeneic groups (not shown) did not show detectable signs of cellular infiltration or rejection. Histology of graft hearts from the syngeneic group were comparable to the alymphoid specimens at both day 1 and 7. These results show no histological evidence of rejection in any of the groups at day 1, although focal infiltrates were present. At day 7, the allogeneic, but not the alymphoid or syngeneic, group had rejection based on myocyte necrosis and extensive mononuclear cell infiltrates.

To determine the composition of the cellular infiltrate, we analysed the gene expression of cell surface markers of T cells (TCRoad, TCRα, CD3ϵ, CD4, CD8α, CD8β), B cells (CD19), macrophages (F4/80), and leucocytes (CD45) in graft heart

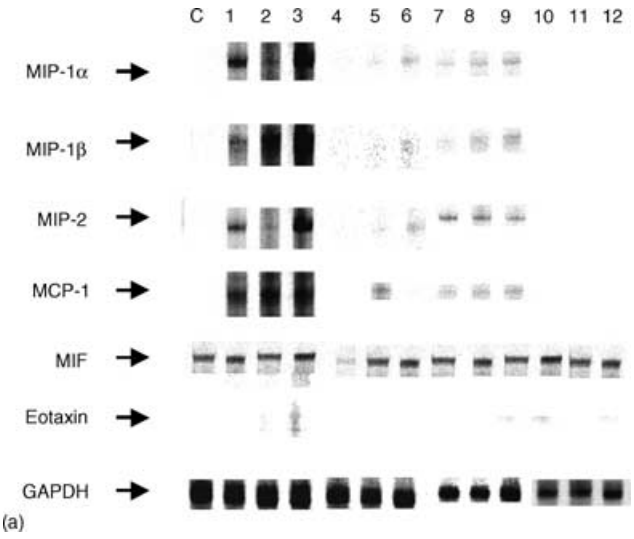

Figure 3. Early expression of chemokines. (a) Total RNA was isolated by RPA with ^{35}S-labelled template including MIP-1α, MIP-1β, MIP-2, MCP-1, MIF, and eotaxin. Untransplanted control heart (lane C), allogeneic graft hearts d 3,5,7 (lanes 1–3), alymphoid graft hearts d 3,5,7 (lanes 4–6), syngeneic graft hearts d 3,5,7 (lanes 7–9), and allogeneic native hearts d 3,5,7 (lanes 10–12). Densitometry analysis was performed using ImageQuant software (Molecular Dynamics), and expression in control (open), day 3 (checks), day 5 (filled) and day 7 (crosshatch) of (b) MIP-1α (c) MIP-1β (d) MIP-2 (e) MCP-1 (f) MIF, and (g) eotaxin were normalized to the housekeeping gene GAPDH. The data shown are representative of three independent experiments.

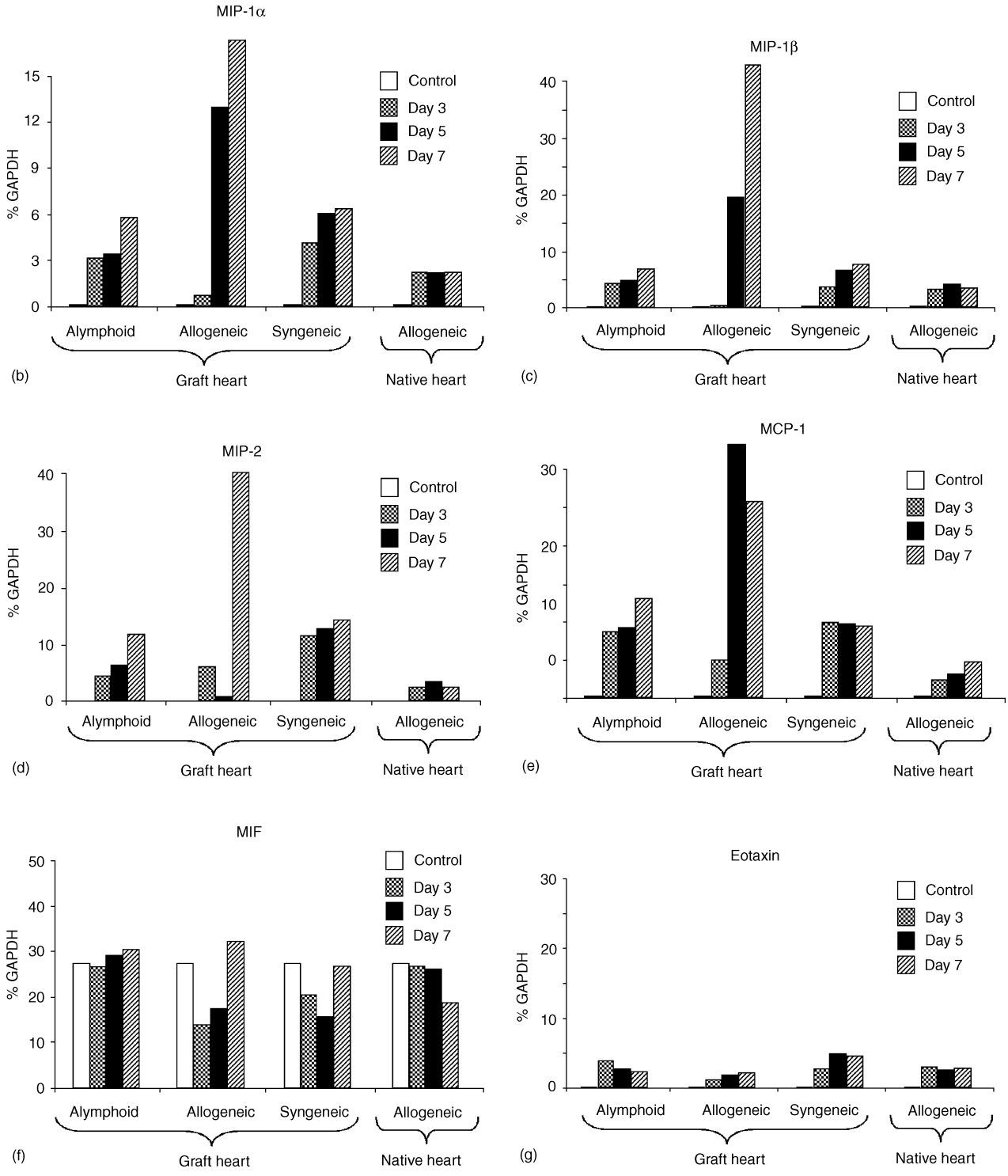

Figure 3. continued

tissue at 3, 5, and 7 days following transplantation by RPA (Fig. 5). Both the alymphoid and syngeneic groups showed increased expression of F4/80 and CD45 genes, indicating infiltration by macrophages and leucocytes (Fig. 5c, d). These markers remained elevated thru day 7 post transplantation. As expected, the allogeneic group showed increasing levels of TCRα and CD8α indicating infiltration by CD8αβ T lymphocytes in addition to macrophages and leucocytes (Fig. 5b). We also detected increased expression of CD3ϵ and CD8β confirming the increased infiltration by $TCR\alpha^+$ $CD8^+$ lymphocytes

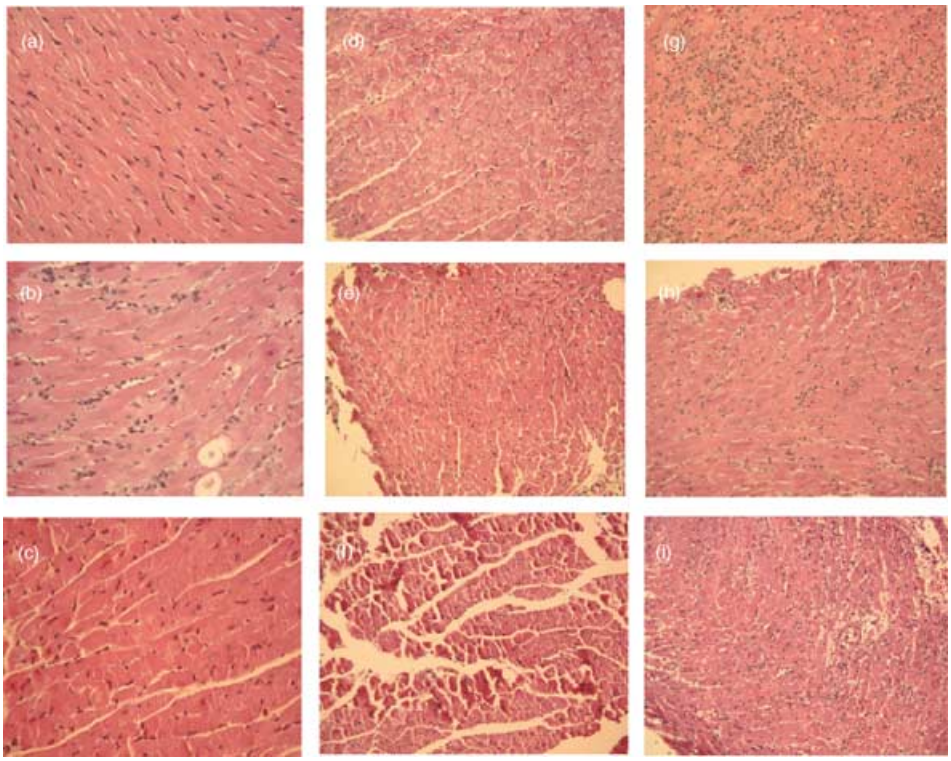

Figure 4. Histology. (a) Analysis of untransplanted control BALB/c heart (b) allogeneic native heart d 3 (c) allogeneic native heart d 7 (d) allogeneic graft heart d 3 (e) alymphoid graft heart d 3 (f) syngeneic graft heart d 3 (g) allogeneic graft heart d 7 (h) alymphoid graft heart d 7 (i) syngeneic graft heart d 7. Donor and native hearts were removed at 3 and 7 days after transplantation and stained with haematoxylin and eosin. Multiple sections were examined for each heart and the extent of rejection quantified on a scale of 0–4 using a modified International Society Heart Transplantation grading scale.

(not shown). Only low levels of CD4, CD19, or TCR gene expression were detected showing a paucity of CD4 T, γδ T, or B cells in the grafts. These results demonstrate that macrophage and leucocyte infiltration occurred in all groups, but high level lymphocytic infiltration was present only in the allogeneic group.

To analyse the effect of cellular recruitment by chemokines, we performed a kinetic analysis of chemokine receptor expression in graft hearts from day 3 through day 7 after transplantation in the alymphoid, allogeneic, and syngeneic groups (Fig. 6). In the allogeneic group, our results show marked up-regulation of the gene expression of multiple chemokine receptors including CCR1, CCR2, CCR5, and CXCR4, and to a lesser extent, CCR1β, CCR3, CCR4, and CXCR3; however, CCR6, CCR8α, CCR8β, CXCR2, and CXCR5 were not up-regulated (Fig. 6b). For all of these receptors maximal levels of expression occurred at day 5 or 7 following transplantation suggesting that the development of the allospecific adaptive immune response amplified the expression of these receptors. In contrast, in the alymphoid and syngeneic groups analysed at the same time points, only CCR2, CCR5, and CXCR4 were markedly increased (Fig. 6c, d, respectively). The striking observation in these results is that CCR1 is up-regulated only in the presence of an adaptive immune response. In addition, there were slightly greater increases in the expression of CCR1β, CCR3, CCR4, and CXCR3 in the allogeneic group as compared to either the alymphoid or syngeneic groups.

To determine the statistical significance of the differential gene expression, 2-factor ANOVA was performed and significance determined by *F*-test (Table 2). Analysis of gene expression in the native heart samples from the syngeneic, alymphoid, and allogeneic recipients did not detect significant changes of expression for any of the parameters analysed. In contrast, analysis of graft hearts in the same three groups showed two main patterns of gene expression. One pattern included genes up-regulated in all three groups (syngeneic, alymphoid, and allogeneic). This subset included MIP-2, MCP-1, CCR1, CCR3, transforming growth factor (TGF)β3, and F4/80. Some genes including IL-1β, IL-1RA, IL-6, MIP-1β, CCR2, CCR4, CCR5, CXCR4, and TGFβ1 showed significant expression in either the syngeneic or alymphoid recipients, plus a trend toward significance in the other group. Another subset including TNF-α, lymphotoxin-α (LT-α), Ltn, RANTES, MIP-1α, TCRα, CD3ϵ, CD8α, and CD8β were significantly up-regulated only in the allogeneic group.

DISCUSSION

The differential contributions of innate and adaptive immunity to the process of allograft rejection remains incompletely understood.[20] A recent report from our laboratory analysing the innate immune response following vascularized heterotopic heart transplantation in both syngeneic and alymphoid recipients

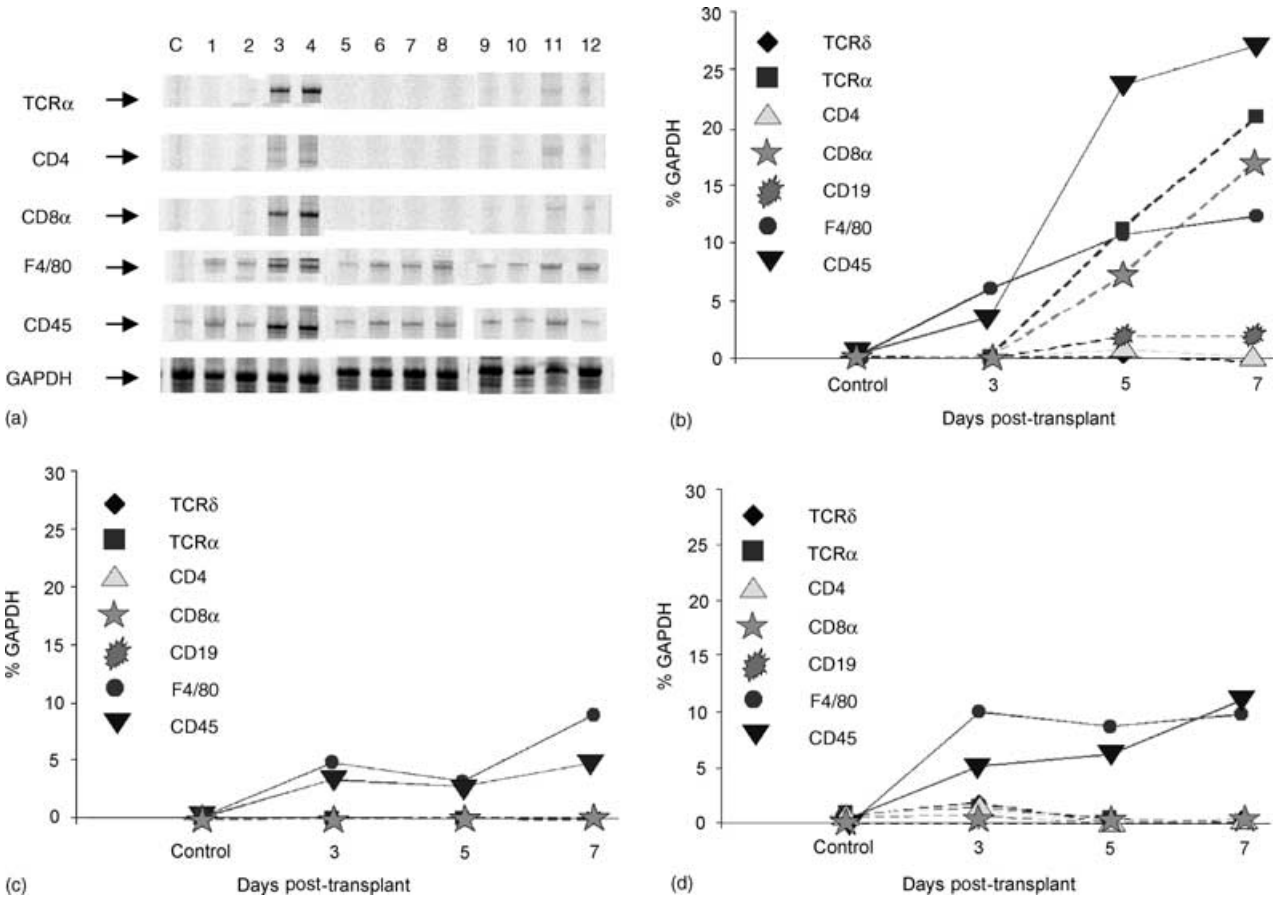

Figure 5. Markers of infiltrating cells. (a) RPA analysis of TCRα, CD4, CD8α, F4/80 and CD45 in untransplanted control heart (lane C), allogeneic graft hearts d 1,3,5,7 (lanes 1–4), alymphoid graft hearts d 1,3,5,7 (lanes 5–8), and syngeneic graft hearts d 1,3,5,7 (lanes 9–12). Densitometry analysis was performed using ImageQuant software (Molecular Dynamics), and expression of TCRδ, TCRα, CD3ϵ, CD4, CD8α, CD19, F4/80 and CD45 quantitated in (b) allogeneic (c) alymphoid, and (d) syngeneic groups. The data shown are representative of three independent experiments.

demonstrated a robust innate response composed of serum acute phase cytokines, intragraft chemokines, cytokines, and chemokine receptors, and infiltration of leucocytes and macrophages.[15] These results were confirmed and extended in the current study with significant increases in serum cytokines (IL-1β and IL-6), intragraft chemokines (MCP-1 and MIP-2), receptors (CCR1, 2, 3, 4, 5 and CXCR4), and cellular markers (CD45 and F4/80). These observations suggest that the antigen independent response has the potential to produce potent proinflammatory mediators and recruit leucocytes and macrophages to the graft. A previous study analysing chemokine up-regulation in syngeneic recipients, also in the heterotopic heart model, similarly detected increased expression of IL-1β and MCP-1; however, this study did not detect increased MIP-2 and did not analyse serum acute phase cytokines, cellular markers, chemokine receptors, or additional cytokines.[21] The differences between the MIP-2 results is likely due to the time of analysis. Our previous results showed maximal expression in the syngeneic and alymphoid recipients of MIP-2 at day 1 following transplantation, but decreased expression by day 3, the earliest time point analysed in the other study. This is shown in our data as low levels of MIP-2 on days 3 and 5, but increased levels on day 7. Another report analysing chemokine expression in syngeneic recipients of skin grafts also detected increased IL-1β, MCP-1, and MIP-2; in addition, they found increased MIP-1α and MIP-1β.[22] Our study also detected a trend for increased MIP-1α and MIP-1β in syngeneic and alymphoid recipients supporting the possibility that these chemokines are induced in the absence of adaptive immunity.[15] Previous studies have demonstrated the importance of chemokine and chemokine receptors in the rejection process. Deficiency of CCR1, CCR5, or CXCR3, which were all up-regulated in our studies by antigen independent mechanisms, all prolong graft survival.

There have been numerous studies analysing inflammatory and immune parameters during allograft rejection. Our focus in this study was to identify subsets of these markers expressed only in the context of an allogeneic response. Not surprisingly, we detected T-cell markers including TCRα, CD3ϵ, CD8α, and CD8β that were increased in the allogeneic grafts. CD8α can be expressed on other cell types, for example dendritic cells, however, comparable increases of all four markers within allografts strongly suggests infiltration by CD8 T cells. Interestingly, we detected low levels of CD4 suggesting discordance between mRNA and protein expression or low level infiltration

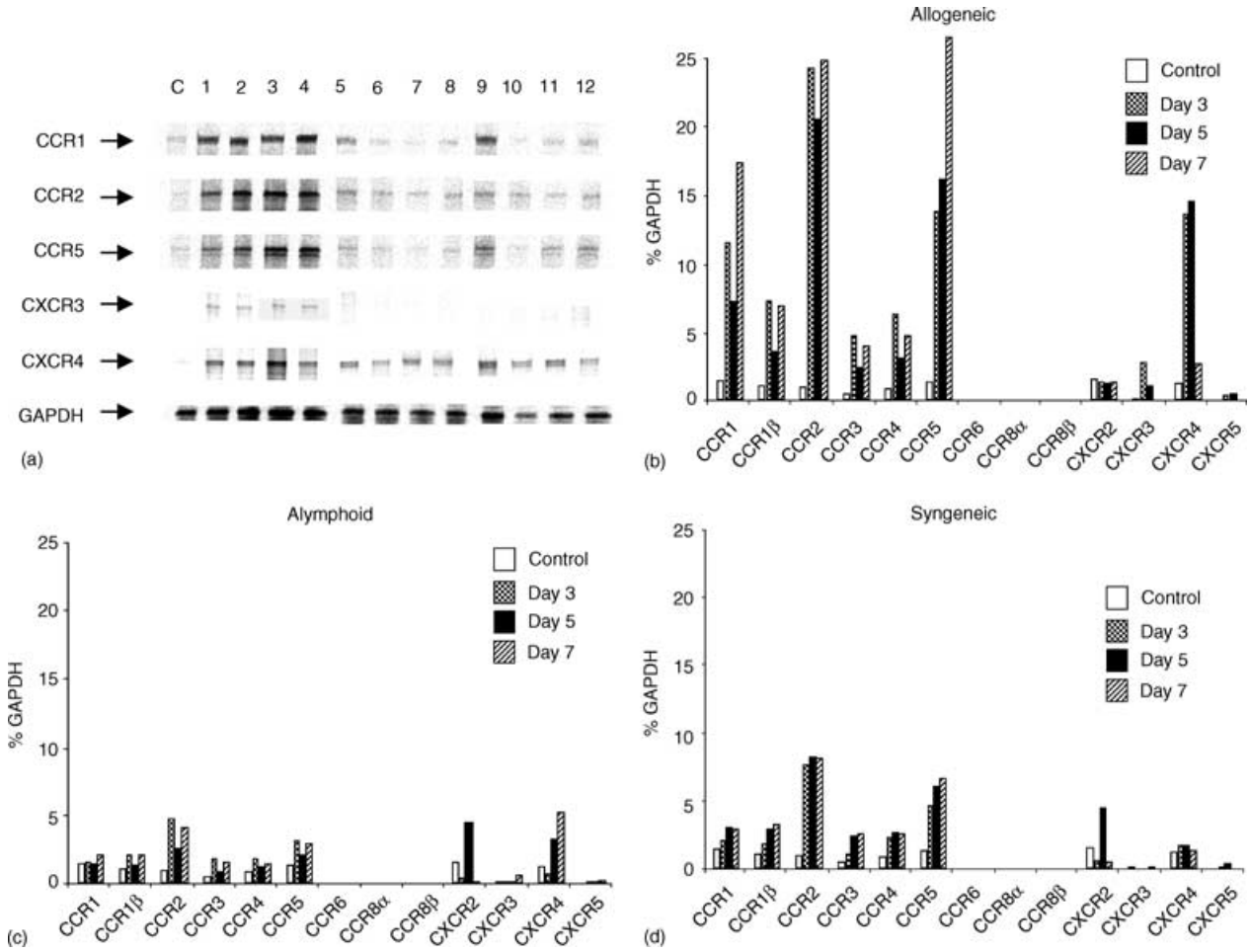

Figure 6. Chemokine receptor expression. (a) RPA analysis of CCR1, CCR2, CCR5, CXCR3, and CXCR4 in untransplanted control heart (lane C), allogeneic graft hearts d 1,3,5,7 (lanes 1–4), alymphoid graft hearts d 1,3,5,7 (lanes 5–8), and syngeneic graft hearts d 1,3,5,7 (lanes 9–12). Densitometry analysis was performed using ImageQuant software (Molecular Dynamics), and expression of CCR1, CCR1b, CCR2, CCR3, CCR4, CCR5, CCR6, CCR8a, CCR8b, CXXCR2, CXCR3, CXCR4, and CXCR5 in (b) allogeneic (c) alymphoid, and (d) syngeneic groups. The data shown are representative of three independent experiments.

by CD4 T cells; however, this does not exclude a crucial role for CD4 T cells, for example, in the peripheral lymphoid tissues. Previous studies have shown correlations between rejection and products of CD8 T cells important in cytotoxicity including granzyme B, perforin and granulysin,[23,24] suggesting an effector function for CD8 T cells in rejection.

Our analysis of serum cytokines showed that IFN-γ was up-regulated only in the allogeneic group. In addition, IL-6, which was expressed at day 1 in all groups, was up-regulated at day 7 only in the allogeneic group. Interestingly, IFN-γ was reproducibly maximally expressed at day 5, with decreased levels evident by day 7. Consistent with this observation, IP-10 was also maximally increased within the graft on day 5. Also, IFN-γ mRNA from allografts was maximally expressed at day 5, suggesting that IFN-γ was produced, at least in part, by intragraft sources. We also detected the T helper 1 cytokines TNF-α and LT-β only within the allogeneic group. In addition, the chemokines Ltn, RANTES, and IP-10 were up-regulated only in the allogeneic group. Previous reports have shown induction of IP-10 in graft endothelial cells following transplantation.[25] However, the template used in our RPA analyses does not detect the BALB/c donor allele; therefore IP-10 may also be expressed at early time points in the non-allogeneic groups. Interestingly, all chemokines and cytokines detected only in the allogeneic group were up-regulated at late time points, for example days 5 or 7, in our kinetic analysis consistent with the increased time necessary to generate an adaptive immune response. In contrast, the factors up-regulated only in the syngeneic and alymphoid groups were induced at early time points, for example days 1 or 3, consistent with rapid induction by an innate response. Interestingly, analysis of serum cytokines with hierarchical clustering algorithms effectively differentiated the allogeneic group from the syngeneic and alymphoid groups.

Taken together, our results detected multiple parameters that were only expressed in the allogeneic group. However, many of these have been reported to be produced independently of adaptive immunity. For example, IFN-γ is produced by natural killer (NK) cells[26] TNF-α by macrophages and other cell

Table 2. Analysis of variance

		Control		Syngeneic NH			Alymphoid NH			Allogeneic NH			Syngeneic GH			Alymphoid GH			Allogeneic GH		
		Mean	Variance	Mean	Variance	*P*	Mean	Variance	*P*	Mean	Variance	*P*	Mean	Variance	*P*	Mean	Variance	*P*	Mean	Variance	*P*
Cytokines	IL-1α	0·6	0·3	0·9	0·4	NS	1·0	0·1	NS	0·2	0·0	NS	0·9	0·1	NS	0·8	0·4	NS	1·2	1·9	NS
	IL-1α	0·6	0·2	1·1	1·0	NS	2·2	0·3	NS	1·3	0·3	NS	3·4	0·7	NS	3·4	5·6	0·0	26·9	562·4	0·0
	IL-1RA	0·5	0·2	1·1	0·7	NS	1·3	0·3	NS	0·9	0·3	NS	3·8	0·8	NS	3·7	0·2	NS	16·5	220·1	0·0
	IL-2	0·0	0·0	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·1	0·0	NS	1·2	0·1	NS	0·6	0·8	NS
	IL-3	0·0	0·0	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS
	IL-4	0·0	0·0	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·2	0·0	NS	0·0	0·0	NS	0·1	0·0	NS
	IL-5	0·0	0·0	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS
	IL-6	0·5	0·1	1·3	0·7	NS	1·4	0·9	NS	1·0	0·3	NS	0·9	0·6	NS	2·4	1·9	0·0	9·0	15·1	0·0
	IL-9	0·0	0·0	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS
	IL-10	0·4	0·1	0·7	1·0	NS	0·3	0·1	NS	0·8	0·5	NS	0·4	0·1	NS	0·7	0·2	NS	0·7	0·3	NS
	IL-12p35	0·1	0·0	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·6	0·1	NS	0·0	0·0	NS	0·0	0·0	NS
	IL-12p40	0·4	0·2	1·1	0·2	NS	1·0	1·0	NS	0·7	0·2	NS	1·7	1·6	NS	0·4	0·2	NS	0·4	0·1	NS
	IL-13	0·0	0·0	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS
	IL-15	4·2	1·6	5·2	1·3	NS	2·5	0·6	NS	6·4	3·4	NS	2·2	1·2	NS	0·9	0·3	NS	1·0	0·2	NS
	IL-18	1·0	0·6	0·7	0·8	NS	0·8	0·7	NS	0·6	0·3	NS	1·5	0·2	NS	2·0	0·3	NS	1·1	0·6	NS
	IFN-γ	0·0	0·0	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS
	IFN-γ	0·8	0·8	1·1	0·2	NS	0·6	0·3	NS	1·3	0·1	NS	0·2	0·1	NS	1·8	0·7	NS	6·8	70·9	0·0
	TNF-α	0·1	0·0	1·1	0·5	NS	0·5	0·3	NS	0·6	0·2	NS	2·0	1·2	NS	0·7	1·1	NS	6·4	33·1	0·0
	LT-α	0·1	0·0	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·4	0·1	NS	0·0	0·0	NS	0·9	1·0	NS
	LT-β	0·7	0·3	0·9	0·5	NS	0·0	0·0	NS	0·8	0·1	NS	0·3	0·0	NS	0·2	0·0	NS	2·5	6·3	0·0
	TGF-β1	1·4	0·2	2·0	1·2	NS	2·9	1·4	NS	2·2	1·3	NS	4·8	4·5	0·0	1·2	0·4	NS	5·1	9·3	0·0
	TGF-β2	1·0	1·1	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	1·8	1·0	NS	2·3	0·8	NS	1·7	0·5	NS
	TGF-β3	3·9	0·9	6·6	2·7	NS	5·4	1·8	NS	4·6	1·0	NS	6·7	13·1	0·0	11·8	28·6	0·0	5·2	5·6	0·0
Chemokines	Ltn	0·4	0·1	0·4	0·0	NS	0·3	0·0	NS	0·3	0·0	NS	0·2	0·1	NS	0·4	0·1	NS	14·7	346·1	0·0
	RANTES	0·4	0·0	0·4	0·0	NS	0·3	0·0	NS	0·4	0·1	NS	0·9	0·5	NS	1·6	2·5	NS	63·5	5501·8	0·0
	IP-10	1·7	0·2	3·1	0·2	NS	2·2	0·4	NS	1·9	0·6	NS	0·6	0·2	NS	0·6	0·3	NS	23·2	747·4	0·0
	Eotaxin	0·7	0·6	0·4	0·0	NS	0·3	0·0	NS	0·4	0·0	NS	0·1	0·0	NS	0·3	0·0	NS	0·6	0·1	NS
	MIP-1α	1·9	1·0	2·6	0·2	NS	2·1	0·0	NS	2·8	1·2	NS	0·8	0·3	NS	0·8	0·7	NS	7·6	64·2	0·0
	MIP-1β	2·2	0·7	3·6	0·2	NS	2·2	0·3	NS	2·4	1·3	NS	1·7	1·0	NS	1·6	1·2	NS	13·2	164·6	0·0
	MIP-2	1·8	0·4	3·0	1·5	NS	2·3	0·2	NS	2·8	0·4	NS	6·1	37·8	0·0	3·3	11·2	0·0	14·1	154·4	0·0
	MCP-1	2·9	0·6	1·9	0·9	NS	2·2	0·5	NS	1·4	0·2	NS	3·0	8·8	0·0	5·6	31·4	0·0	20·2	165·8	0·0
Receptors	CCR1	0·7	0·3	0·4	0·1	NS	0·6	0·1	NS	0·3	0·1	NS	4·2	26·5	0·0	4·1	2·1	0·0	9·5	12·8	0·0
	CCR1β	0·7	0·6	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	3·2	10·9	0·0	4·4	0·6	NS (.114)	5·4	2·3	0·0
	CCR2	1·2	0·5	2·1	0·5	NS	2·3	1·0	NS	1·7	2·1	NS	4·5	31·7	0·0	6·6	0·9	NS (.067)	18·6	9·9	0·0
	CCR3	0·3	0·2	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	3·1	6·2	0·0	2·6	1·3	0·0	2·3	2·0	0·0
	CCR4	0·4	0·3	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	4·0	9·5	0·0	3·8	1·3	NS	4·6	1·1	0·0
	CCR5	1·5	1·1	1·7	0·7	NS	1·4	0·5	NS	0·7	1·1	NS	4·3	35·1	NS (0·085)	5·5	0·3	0·0	17·0	2·8	0·0
	CCR6	0·0	0·0	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS
	CCR8α	0·3	0·1	0·3	0·1	NS	0·3	0·1	NS	0·3	0·1	NS	0·0	0·0	NS	0·0	0·0	NS	0·0	0·0	NS
	CXCR2	0·7	0·4	1·1	0·5	NS	0·8	0·5	NS	1·1	0·5	NS	0·5	0·0	NS	2·1	13·3	NS	1·1	0·2	NS
	CXCR3	0·2	0·1	0·2	0·1	NS	0·2	0·1	NS	0·2	0·1	NS	0·0	0·0	NS	0·2	0·1	NS	1·2	1·4	NS
	CXCR4	0·5	0·3	0·7	0·3	NS	0·8	0·4	NS	0·9	0·6	NS	1·4	0·1	NS	2·6	4·3	0·0	10·8	30·2	0·0
	CXCR5	0·2	0·0	1·0	1·1	NS	0·6	0·7	NS	0·7	0·7	NS	0·1	0·0	NS	0·1	0·0	NS	0·2	0·0	NS

Markers																				
	TCRδ	0·1	0·0	0·1	0·0	0·0	NS	0·0	NS	0·2	0·1	NS	0·7	0·7	NS	0·0	0·0	NS	0·1	0·0	NS
	TCRα	0·3	0·1	0·3	0·1	0·1	NS	0·3	NS	0·4	0·3	NS	0·8	0·3	NS	0·0	0·0	NS	8·0	101·1	0·0
	CD3ϵ	0·2	0·0	0·1	0·0	0·0	NS	0·1	NS	0·4	0·2	NS	0·7	0·6	NS	0·0	0·0	NS	6·3	61·8	0·0
	CD4	0·2	0·0	0·2	0·0	0·0	NS	0·3	NS	0·2	0·0	NS	0·7	0·3	NS	0·0	0·0	NS	0·2	0·2	NS
	CD8α	0·2	0·0	0·1	0·0	0·0	NS	0·2	NS	0·5	0·3	NS	0·8	0·6	NS	0·0	0·0	NS	6·2	63·4	0·0
	CD8β	0·1	0·0	0·1	0·0	0·0	NS	0·2	NS	0·3	0·2	NS	0·4	0·6	NS	0·0	0·0	NS	5·7	55·0	0·0
	CD19	0·1	0·0	0·2	0·0	0·0	NS	0·2	NS	0·6	0·3	NS	0·0	0·0	NS	0·0	0·0	NS	1·0	1·2	NS
	F4/80	0·6	0·1	1·1	0·1	1·3	NS	1·9	NS	1·2	0·1	NS	4·9	17·8	0·0	4·6	10·0	0·0	8·3	14·8	0·0
	CD45	1·5	2·2	0·7	0·1	0·2	NS	0·9	NS	0·8	0·2	NS	2·6	5·5	NS	3·1	2·0	NS	15·0	145·2	0·0

Multiple parameters including cytokines, chemokines, receptors, and cellular markers were analysed by 2-factor ANOVA for graft and native hearts in the syngeneic, alymphoid, and allogeneic groups. Significance between each experimental group and control was determined for $P < 0{\cdot}05$ by F-test. Additional parameters not shown in previous figures are included

types[27] and LT-β by B cells.[28] Although Ltn and RANTES are highly produced by T cells, Ltn is also produced by NK and mast cells,[29,30] and RANTES is also produced by endothelial cells and basophils[31] and IP-10 is produced by endothelial cells.[25] Thus, although these parameters were detected in our study only in the context of allograft rejection, in a different milieu they may be expressed independently of adaptive immunity. This observation suggests extensive functional overlap in the regulation and utilization of parameters such as IFN-γ, TNF-α, LT-β, Ltn, RANTES, and IP-10 by the innate and adaptive responses. This concept is strongly supported by another observation based on our results. All 13 of the parameters that were induced by innate mechanisms in the syngeneic and alymphoid groups (IL-1β, IL-1RA, IL-6, MIP-2, MCP-1, CCR1, CCR2, CCR3, CCR4, CCR5, CXCR4, F4/80, CD45) showed amplified expression in the allogeneic group suggesting positive feedback by the adaptive to the innate response. For many parameters there may not be distinct demarcations between the innate and adaptive components of the response, rather, factors may participate in and be regulated by both components. Thus, the only definitive qualitative distinction between the innate and adaptive responses in our studies is CD8 T cells, whereas many of the factors produced or regulated by T cells may be components of both responses, although quantitative increases in expression were apparent in the allogeneic group that generated an adaptive response.

Our experimental design incorporated both a syngeneic and alymphoid group to dissect the innate contribution to the rejection response. The syngeneic group has normal lymphocyte components, but lacks alloantigen. Because of genetic identity, NK cells should not be activated; however, natural antibodies could be activated in response to previously sequestered antigens.[32] Conversely, the alymphoid group lacks functional T and B lymphocytes and could not generate responses by natural antibodies; however, because of major histocompatibility complex class I and other diffferences, NK cells could be stimulated. When compared to gene expression in untransplanted control hearts, different degrees of significance were calculated for the syngeneic and alymphoid groups; however, because of similar trends in both groups, the only significant difference in a direct comparison of the syngeneic vs. alymphoid groups was for CXCR4 ($P < 0{\cdot}01$). Previous reports have shown that CXCR4 is expressed weakly on NK cells, as well as most other cell types, supporting the possibility of NK cell infiltration in the alymphoid group.[33] However, assuming NK cell infiltration, our results did not detect modulation of any additional parameters analysed in our study. A previous study analysing NK cells in skin grafts did not detect responses in the BALB > B6 group, the strain combination used in our protocol, although they did detect response in the reciprocal combination.[22] As we did not detect evidence of NK cell modulation of any of the parameters analysed in our study, we did not directly analyse NK cell infiltration. Also, we did not detect evidence of modulation of any parameters by natural antibodies. Nevertheless, we cannot exclude the possibility that natural antibodies or NK cells modulated additional parameters not analysed in our study. It will be important in future studies to determine the components of innate immunity activated following transplantation and their roles in promoting rejection.

Previous studies have established that adaptive immunity, in particular T cells, are necessary for allograft rejection. Our current study has identified multiple parameters including chemokines, cytokines, receptors, and cellular markers that are up-regulated in the allogeneic, but not syngeneic or alymphoid, groups. In contrast, an additional subset of parameters is induced by antigen-independent mechanisms in the syngeneic and alymphoid groups; however, all of these parameters, which are induced by innate mechanisms, are further up-regulated in the allogeneic group. Importantly, these observations suggest extensive regulatory interactions and functional overlap between the innate and adaptive components of the rejection response.

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